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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Ariel Ruiz i Altaba

EXAMINER: Gary B. Nickol

SERIAL NO.: 09/825,155

ART UNIT: 1642

Filed: April 3, 2001

For: METHODS AND MATERIALS FOR THE DIAGNOSIS AND
TREATMENT OF SPORADIC BASAL CELL CARCINOMADECLARATION UNDER 37 C.F.R. 1.132ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

I, ARIEL RUIZ I ALTABA, hereby declare and state that:

1. I am an Assistant Professor, in the Department of Cell Biology at the New York University School of Medicine, having received my Ph.D. degree from Harvard University in 1989. After that I was a postdoctoral fellow at the college of physicians and surgeons of Columbia University. My full curriculum vitae is attached hereto as Exhibit A.

2. My principal area of research is development of the vertebrate brain and tumorigenesis, and among other positions I serve as reviewer in numerous funding agencies of many countries, including the US (NIH, NSF, American Brain Tumor Foundation), France, Italy, Spain, UK, Austria, The Netherlands. I also have served as reviewer for numerous scientific journals including Nature, Science, Neuron, Nature Genetics, Nature Cell Biology, Development, Genes and Development. I am a member of the editorial board of Mechanisms of Development and a member of the Society for Neuroscience and the Society for Developmental Biology.

3. In the course of my activities, I have been listed as inventor on several patent applications, including the one noted above entitled "METHODS AND MATERIALS FOR THE DIAGNOSIS AND TREATMENT OF SPORADIC BASAL CELL CARCINOMA", having U.S. Serial Number 09/825,155, which claims priority to U.S. application Serial Number 09/102,491, filed on June 22, 1998, which claims the benefit of priority to U.S. provisional application Serial Number 60/050,286, filed on June 20, 1997.

4. I have reviewed the disclosure of the present application, with particular emphasis on the identification of subject matter that could directly be applied to therapeutic implications, and more specifically on pharmaceutical compositions useful for the treatment of cellular debilitation, derangements and/or dysfunctions and/or other disease states in mammals caused by the development of sporadic basal cell carcinoma, with particular emphasis on compositions comprising therapeutically effective amounts of inhibitors of Gli1 expression and/or function. In my opinion, the disclosures of this current application are sufficient to enable one skilled in the art to make or use the invention described therein and concomitantly provide to the practitioner teachings that could be applied for the indicated therapeutic purposes. Furthermore, I have provided references attached herein as Exhibit B that support the therapeutic use of small interfering RNA molecules. Thus, a skilled artisan would be able to practice the current invention given the level of knowledge in the art at the time the invention was made.

5. In this regard, and in corroboration of the disclosure of this current application, I have conducted various experiments in my own laboratory to further confirm the disclosure in this application and which supports the utility of small molecule antagonists of Gli1 for inhibition of tumor cell proliferation. Particularly, my laboratory has conducted experiments using small interfering RNA molecules (siRNA) that antagonize Gli1 expression and function, which at the same time inhibit the proliferation of tumor cells *in vitro*. Our *in vitro* models predict reliable tumor growth as the parameters measured are cell division per se, not morphological changes, changes in invasiveness or behavioral. siRNAs against Gli1 inhibit tumor growth and we have no reason to think that this will not be true *in vivo*. These assays are now underway.

6. The studies conducted under my supervision which provide evidence for the effect of inhibitors of Gli1 activity /function on tumor cell proliferation/growth are summarized as follows:

a.) A small molecule inhibitor of Gli1 activity/function was developed. The small molecule inhibitor of Gli1 activity /function that was studied in my laboratory was a small interfering RNA molecule. This molecule was prepared by the following method: the siRNAs are designed based on the available sequence of the Gli genes. The chemical synthesis is made by a commercial vendor (in our case Dharmacon, Inc.) Proliferation is measured in two ways. 1.) Through the incorporation of bromodeoxyuridine (BrdU) in the DNA of cycling cells, which is then detected with specific anti-BrdU antibodies in single cell manner microscopically. This assay directly measures DNA replication and thus cell division and proliferation. 2.) Through the methylthiazoletetrazolium (MTT) assay, which measures the metabolic activity of the cells mitochondria in a plate reader. This second assay measures metabolic activity and thus changes in cell number and viability. The figure as shown herein was done with method 1.)

b.) The tumor cells tested included primary human glioma, medulloblastoma and glioblastoma tumor cells as well as the U87 human glioma tumor cells and human Daoy medulloblastoma cells. Tests with BCC cell lines are difficult because there are no reliable BCC cell lines available. However, we think that tumor cells exemplify the action of SHH-Gli signaling in those tumors that require this pathway for proliferation as the same principle applies to both skin and brain tumors: they are both derived from epithelial sheets and both use the SHH-Gli pathway for normal proliferation. All studies done *in vivo* and *in vitro* with BCCs and brain tumors show that inhibition of SHH-Gli signaling will cause a cessation of proliferation and thus inhibition of tumor growth of BCCs and brain tumors that depend of SHH-Gli signaling for proliferation.

c.) The results as shown herein, provide proof that Gli1 plays a role in tumor cell proliferation and/or growth, since blocking the expression/activity/function of Gli1 with small RNA molecule antagonists inhibit tumor cell proliferation *in vitro*. This result is fully supported by our data on the inhibition of brain tumor growth by

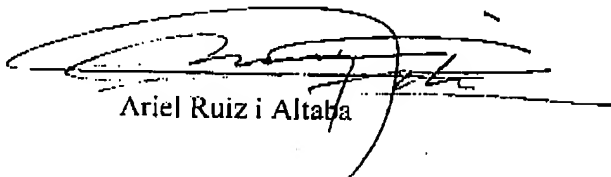
cyclopamine (a plant alkaloid that inhibits the action of Smoothened, a protein that mediates the HH signal at the membrane) that we originally reported (Dahmane et al., 2001). Tumors that are inhibited by siRNAs against Gli1 are also inhibited by cyclopamine.

7. The results shown here demonstrate that development of pharmaceutical compositions containing inhibitors/antagonists of Gli1 expression/activity/function is an attractive strategy for use as an anti-tumor therapy

The advantages would be i) the specificity of the treatment to Gli function as opposed to the non-specific action of chemotherapeutic agents that inhibit the proliferation and viability of any dividing cell. ii) the minor side effects that are expected from systemic administration as in children or adults the SHH-Gli1 pathway is involved in homeostatic aspects that are not vital, such as maintenance of the gastric mucosa (van den brink et al., 2000). Thus temporary arrest of these other functions would not cause severe damage. iii) easier administration than gene therapy as siRNAs should not provide a lasting effect in the genome. iv/ in mice Gli1 is dispensable for normal development of cells and tissues (its function being compensated by the other Glis and if it is so in humans, the side effects of its inhibition in the tumors will be nil. v) siRNAs can be easily administered in quantifiable doses either systemically or locally.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18 of the U.S. Code, Section 1001, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Dated: Feb 11th, 2003


Ariel Ruiz i Altaba

MATERIALS AND METHODS

Cell culture and siRNAs transfections

U87 glioblastoma cells were cultured in MEM with 10% fetal bovine serum (FBS), and glioblastoma primary cultures were cultured in DMEM/F12 with 10% FBS. For transfection of siRNAs, cells were plated the day before the treatment in p16 well plates, at a 70% cell density. 24h later cells were transfected using the Oligofectamine reagent from Gibco (cat# 12252-011) following the specifications of the manufacturer. The final concentration of siRNA was 200nM. Three hours after, the transfection media was changed to normal growing media (10%FBS) and the cells were kept growing for another 48h (or 24h where indicated) before processing.

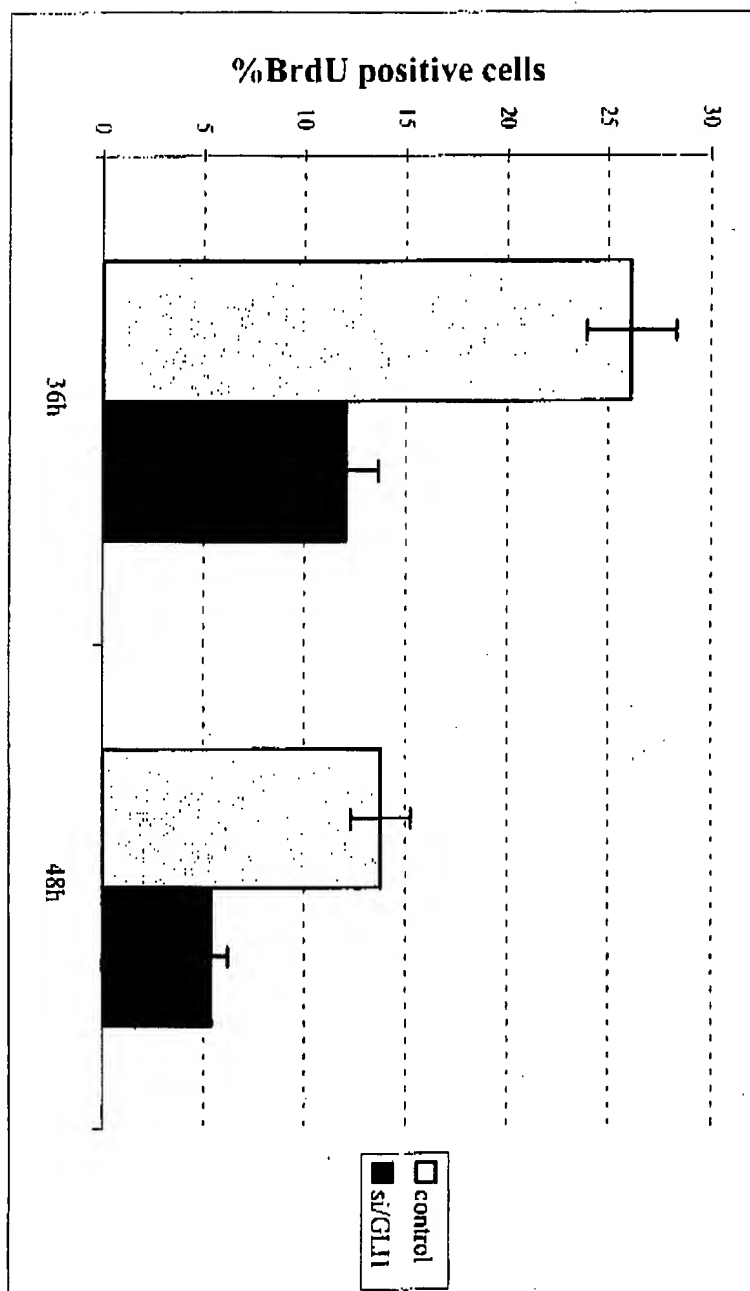
Proliferation assay

BrdU (6 μ g/ml) incorporation was done for 2h in U87 glioblastoma cells and for 14h in the primary glioblastoma cells. After that the cells were fixed for 5 minutes in paraformaldehyde (PFA) 4%. Immunocytochemistry was performed with an anti-BrdU antibody (Becton-Dickinson) and fluorescein-conjugated secondary antibodies (Boehringer Mannheim). The measurement was done by counting percentage of BrdU positive cells per field, counting at least 8 fields per point.

RESULTS

The results showed that both the U87 glioblastoma cells, as well as the primary glioblastoma cells, when transfected with small inhibitory RNA molecules specific for Gli1, showed significant inhibition in proliferative capacity, as compared to control cells.

U87 proliferation assay



Primary tumors proliferation assay

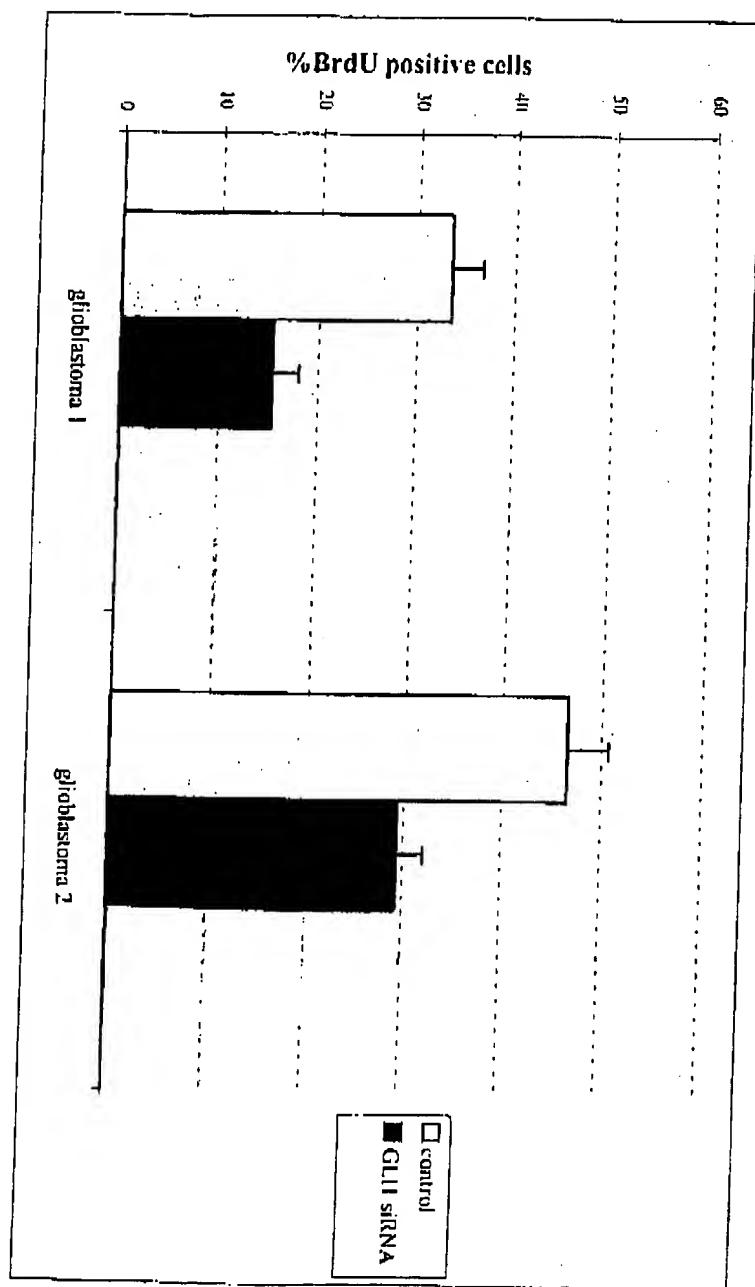


EXHIBIT A
ARIEL RUIZ I ALTABA
CURRICULUM VITAE

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Developmental Genetics Program and Dept. Cell Biology
New York University School of Medicine, 540 First Avenue, New York, N.Y. 10016

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Education

1980 - 1981 First year of University, University of Barcelona Biology Section,
Barcelona, Catalonia, Spain.

1981 - 1982 Second year of College, New York University, New York.

1982 - 1984 Third and Fourth years of College, Columbia University. A.B. Degree
granted.

Undergraduate work

1982 Laboratory of Dr. A. Pellicer, Dept. of Pathology, NYU Medical Center

1982 - 1984 Laboratory of Dr. G.N. Godson, Dept. of Biochemistry, NYU Medical Center.

Graduate School

1984 - 1985 Department of Cellular and Developmental Biology, Harvard University.

1985 - 1989 Laboratory of Dr. D. A. Melton, Department of Biochemistry and
Molecular Biology, Harvard University. Ph.D. granted.

Post-Doctoral Training

1989 - 1993 Laboratory of Dr. T. M. Jessell, Howard Hughes Medical Institute,
Columbia University.

1993 - 1994 Associate Research Scientist, Center for Neurobiology and Behavior,
Laboratory of Dr. T.M. Jessell, Columbia University.

Academic Appointments

1994 - 2000 Assistant Professor, Skirball Institute and Department of Cell Biology,
NYU School of Medicine.

1999- Associate Professor, Skirball Institute, NYU School of Medicine.

Fellowships and Awards

1987 Paul Mazur Fellowship, Harvard University School of Arts and Sciences.

1989-1993 Post-doctoral Fellowship, Howard Hughes Medical Institute.

1994 Young Investigator Award, Mechanisms of Development.

1995 Whitehead Fellowship for Junior Faculty at New York University.

1996 - 2000 The Pew Scholars Program in the Biomedical Sciences.

1997 - 1999 Basil O'Connor Starter Award. March of Dimes Foundation.

2001 - 2005 Hirsch Award

Advisory Boards

- 1994 - Advisory Editorial Board, *International Journal of Developmental Biology*.
2000- Advisory Editorial Board, *Mechanisms of Development*
2000 Scientific Advisory Board, Midwestern Regeneration Center
2001- Board Member, Spanish Society for Developmental Biology
2002- Reviewing Board, Ramón y Cajal Programme, Spanish Foundation for Science and Technology

Professional Affiliation

- 1995 - Society for Developmental Biology.
1996 - Society for Neuroscience.
1997- Spanish Society for Developmental Biology

Teaching Experience

- 1987, 1988 Teaching assistant to Dr. Melton at Harvard University.
1991, 1993, 1995 Invited lecturer in Biologia Molecular de la Cèl·lula: Proliferació, Diferenciació, Organització. Universitat de Barcelona and Universidad Internacional Menéndez Pelayo. Barcelona, Catalonia, Spain
1991 Invited teacher/lecturer in Modern Techniques in Developmental Biology—A Practical Course Oxford, United Kingdom.
1993 Invited teacher/lecturer in Pattern Formation in Early *Xenopus* Embryos. EMBO Course. Utrecht, Holland.
1995- Teaching Embryology, Developmental Biology and Neuroscience at NYU Medical and Graduate Schools.
2003- Lectures and practical labs in the Developmental Genetics Track Graduate Course and at the Biology Department at NYU.

Grant Reviews For NIH committees, NSF, Ramon Y Cajal Foundation, HFSP, National Brain Tumor Association, and several agencies in Italy, Austria, United Kingdom, France, The Netherlands, Canada and Spain.

Paper Reviews For *Nature*, *Neuron*, *Nature Cell Biology*, *Nature Genetics*, *Current Biology*, *Genes and Development*, *Int. J. Dev. Biology*, *Development*, *Mechanisms of Development*, *Developmental Biology*, *Science*, *Mol. Cell. Neurosci.*, *Developmental Cell* and others

Patents and patent applications: Five

Major Research Interests

Molecular mechanisms underlying the development and evolution of the vertebrate brain, from pattern formation to cell type specification and overall growth. Mechanisms of patterning and tumorigenesis by the HH-Gli pathway.

Invited talks, 2000-present:

Pew Meeting, Cozumel, Mexico
 SUNY, Syracuse, New York
 New York Academy of Sciences, New York
 McGill Cancer Center, Montreal, Canada
 Centro de Biología Molecular Severo Ochoa-CSIC, Madrid, Spain
 Centro Superior de Investigaciones Científicas, Barcelona, Spain
 International Society of Developmental Neurobiology, Heidelberg, Germany
 European Teratology Society, Ferrara, Italy
 Max Planck Institute, Berlin, Germany
 Institute Curie and Ecole Normale Supérieure- Paris, France
 UCSF, San Francisco
 Juan March Meeting, Madrid, Spain
 Cajal Institute, Madrid, Spain
 CNS Institute inaugural opening, MRC-King's College, London, UK
 European IIGB meeting, Capri, Italy.
 University of Florence, Italy
 University of Geneva, Switzerland
 Parc Científic, Barcelona, Spain
 ANPP meeting, Paris, France
 II Holoprosencephaly meeting, NIH
 Spring Hippocampal meeting, Cayman Islands
 Pasteur Institute, Paris, France
 1ST Symposium of SFB 590, Duesseldorf, Germany
 Universitat Autònoma de Barcelona, Catalonia, Spain
 Univ. Washington, Seattle, Invited by the neuroscience students
 Pasteur Institute, Paris, France
 King's College, London, UK
 Centre de Regulació Genòmica, Barcelona, Spain
 1ST Latin American Developmental Biology meeting- Chile
 IMP Institute, Vienna, Austria Feb '03
 Meeting on Cancer and Development, Positano, Italy, May '03
 University of Pennsylvania, Philadelphia June '03

Publications

1. Lupski, J., Ruiz Altaba, A. and Godson, G.N. (1984) Promotion, Termination and Anti-termination in the *rpsU-dnaG-rpoD* Macromolecular Synthesis Operon of *E. coli*. *Mol. Gen. Genet.* 195:391-401.
2. Ruiz i Altaba, A., Ozaki, L.S., Zavala, F. and Godson, G.N. (1986) Expression of the *Plasmodium knowlesi* Circumsporozoite Antigen in *E. coli* Directed by *Plasmodium* Bacterial-like Promoters. *Gene* 41:135-144.
3. Ruiz i Altaba, A., Ozaki, L.S., Gwadz, R.W. and Godson, G.N. (1987) Organization and Expression of the *Plasmodium knowlesi* Circumsporozoite Antigen Gene. *Molec. Biochem. Parasitol.* 23:233-245.
4. Ruiz i Altaba, A., Perry-O'Keefe, H. and Melton, D.A. (1987) *Xfin*: an Embryonic Gene Encoding a Multifingered Protein in *Xenopus*. *EMBO J.* 6:3065-3070.

5. Melton, D.A., Ruiz i Altaba, A., Yisraeli, J. and Sokol, S. (1988) Localization and Axis Formation during *Xenopus* Embryogenesis. *Ciba Foundation Symposium Cellular Basis of Morphogenesis. London* 144:16-36.
6. Ruiz i Altaba, A. and Melton, D.A. (1989) Bimodal and Graded Expression of the *Xenopus* Homeobox Gene *Xhox3*. *Development* 106:173-183.
7. Ruiz i Altaba, A. and Melton, D.A. (1989) Involvement of the *Xenopus* Homeobox Gene *Xhox3* in Pattern Formation along the Anterior-Posterior Axis. *Cell* 57:317-326.
8. Ruiz i Altaba, A. and Melton, D.A. (1989) Interaction Between Peptide Growth Factors and Homeobox Genes in the Establishment of Antero-Posterior Polarity in Frog Embryos. *Nature* 341:33-38.
9. Ruiz i Altaba, A. and Melton, D.A. (1990) Axis Formation and the Establishment of Polarity in Frog Embryos. *Trends in Genetics* 6:57-64.
10. Ruiz i Altaba, A. (1990) Neural Expression of the *Xenopus* Homeobox Gene *Xhox3*, Evidence for a Patterning Neural Signal that Spreads Through the Ectoderm. *Development* 108:595-604.
11. Ruiz i Altaba, A. (1991) Vertebrate Development: an Emerging Synthesis. *Trends Genetics* 7:276-280.
12. Ruiz i Altaba, A. and Jessell, T. (1991) Retinoic Acid Modifies Mesodermal Patterning in Early *Xenopus* Embryos. *Genes and Development* 5:175-187.
13. Ruiz i Altaba, A. and Jessell, T. (1991) Retinoic Acid Modifies the Pattern of Cellular Differentiation of the Central Nervous System of Neurula Stage *Xenopus* Embryos. *Development* 112:945-958.
14. Ruiz i Altaba, A., Choi, T. and Melton, D.A. (1991) Expression of the *Xhox3* Homeobox Protein in *Xenopus* Embryos: Blocking its Early Function Suggests the Requirement of *Xhox3* for Normal Posterior Development. *Development Growth and Differentiation*. 33:651-669.
15. Ruiz i Altaba, A. (1992) Cooperation of Planar and Vertical Signals in the Induction and Patterning of the *Xenopus* Nervous System. *Development* 115:67-80.
16. Ruiz i Altaba, A. and Jessell, T. (1992) *Pintallavis*, a Gene Expressed in the Organizer and Midline Cells in *Xenopus*. Involvement in the Development of the Neural Axis. *Development* 116:81-93.
17. Klar, A., Jessell, T.M. and Ruiz i Altaba, A. (1992) Control of Floor Plate Identity and Function in the Embryonic Nervous System. *Cold Spring Harbor Symp. Quant. Biol.* Volume LVII:473-482.
18. Ruiz i Altaba, A. *Xenopus* embryology and molecular biology chapters in *Essential Developmental Biology, A Practical Approach*. C.Stern and P. Holland. Eds. Oxford University Press. Oxford (1993).
19. Ruiz i Altaba, A. (1993) Induction and Axial Patterning of the Neural Plate: Planar and Vertical Signals. *J. Neurobiology* 24:1276-1304.
20. Ruiz i Altaba, A. and Jessell, T.M. (1993) Midline Cells and the Organization of the Vertebrate Neuraxis. *Curr. Op. Gen. and Dev.* 3:633-640.
21. Ruiz i Altaba, A., Cox, C., Jessell, T. and Klar, A. (1993) Deregulated Expression of the Midline Transcription Factor *Pintallavis* Induces Ectopic Expression of a Floor Plate Marker. *PNAS* 90:8268-8272.

22. Ruiz i Altaba, A., Prezioso, V., Darnell, J. and Jessell, T.M. (1993) Sequential Expression of *HNF-3 α* and *HNF-3 β* in embryonic organizing centers: the Node, Notochord and Floor Plate. *Mech. Dev.* 44:91-108.
23. Ruiz i Altaba, A. (1994) Pattern Formation in the Vertebrate Neural Plate. *Trends Neurosci.* 17:233-243.
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26. Ruiz i Altaba, A., Roelink, H. and Jessell, T.M. (1995) Restrictions to Floor Plate Induction by *hedgehog* and Winged Helix Genes in the Neural Tube of Frog Embryos. *Mol. Cell. Neurosci.* 6:106-121.
27. Ruiz i Altaba, A., Placzek, M., Baldassare, M., Dodd, J. and Jessell, T.M. (1995) Early Stages of Notochord and Floor Plate Differentiation Defined by Normal and Induced Expression of *HNF-3 β* in Chick Embryos. *Dev. Biol.* 170:299-313.
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29. Ruiz i Altaba, A. and Théry, C. (1996) Involvement of Livertine, a hepatocyte growth factor family member, in neural morphogenesis. *Mech. Dev.* 60:207-220.
30. Ruiz i Altaba, A. (1997) Catching a Gli-mpse of Hedgehog. *Cell* 90:193-196.
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33. Ruiz i Altaba, A. (1998) Deconstructing the organizer. *Nature* 391:748-749.
34. Nothias, F., Fishell, G. and Ruiz i Altaba, A. (1998) Cooperation of intrinsic and extrinsic signals in the determination of regional identity of the posterior neocortex. *Current Biology* 8:459-462.
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36. Liu, F., Massagué, J. and Ruiz i Altaba, A. (1998) Carboxy-terminally truncated *Gli3* proteins associate with Smads. *Nature Genetics* 20:325-326.
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39. Ruiz i Altaba, A. (1999) *Gli* proteins and Hedgehog signaling in development and cancer. *Trends Genetics* 15:418-425.

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41. Dahmane, N. and Ruiz i Altaba, A. (1999) Sonic hedgehog regulates the growth and patterning of the cerebellum. *Development* 126:3089-3100.
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47. Ruiz i Altaba, A., Gitton, Y. and Dahmane, N. (2001) Embryonic regionalization of the neocortex. *Mech. Dev.* 107:3-11.
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54. Gitton, Y., Dahmane, N., Baik, S., Ruiz i Altaba, A., Neidhardt, L., Scholze, M., Herrmann, B., Kahlem, P., Kahla A., Schrinner, S., Yildirimman, R., Herwig, R., Lehrach, H. and Yaspo, M.L. (2002) A multi-level gene expression analysis of human chromosome 21 orthologs in the mouse: potential insights into Down syndrome. *Nature* 420, 586-590.
55. Stecca, B. and Ruiz i Altaba, A. (2002). Therapeutic potential of modulators of the HH-GLI signaling pathway. *J. Biology*, 1:9.
56. Palma, V., Lim, D., Dahmane, Sanchez, P., Gitton, Y., Alvarez-Buylla, A. and Ruiz i Altaba, A. (2003) Shh-Gli signaling regulates neural stem cell behavior. Submitted.
57. Erich Roessler, Yangzu Du, Jose L. Mullor, Esther Casas, William P. Allen, Ian Ellis, Gabriele Gillesen-Kaesbach, Elizabeth R. Roeder, Jeffrey E. Ming, Ariel Ruiz i Altaba, Maximilian Muenke (2003) Loss-of-

function mutations in the human *GLI2* gene cause holoprosencephaly and familial pan-hypopituitarism. Submitted.

58. Ruiz i Altaba A., Stecca, B. and Sanchez, P. (2003). Shh-Gli signaling in brain tumors: stem cells and developmental programs in cancer. *Cancer Letters-special issue*. In preparation.

Active Funding

Ruiz i Altaba (PI)
NIH/NIHNS
R01 NS37352
Function of the Gli Genes in Sonic Hedgehog Signalling
Role: PI

Ruiz i Altaba (PI)
NIH/NCI
R01 CA78736
Role of the Gli1 in Basal Cell Carcinoma Development
Role: PI

Ruiz i Altaba (PI)
Irma T. Hirschl Trust
The Role of Gli Genes in Brain Tumor Development
Role: PI

Ruiz i Altaba (PI)
Human Frontiers Science Program
Specification and Refinement of Developing Visual Cortex
Role: PI

Post doc grants active in the past two years:

Human Science Frontier's Program, Ramon Areces Foundation, Association Contre le Cancer, American Brain Tumor Society, Pew Latin American Fellowship, Parkinson's Foundation.

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EXHIBIT B